REMARKS

I. Introduction

Applicants respectfully request reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claims 1-30 and 32 are canceled.

Claims 31 and 36 are currently being amended. Exemplary support for the claim amendments is found throughout the specification. For example, support for the phrase "free of human derived proteins and human infectious agents" flows from Applicants' disclosure teaching that the described hPTH is not is not isolated from human sources. Additionally, support for the phrase "does not contain chemically modified amino acids" flows from Applicants' disclosure teaching that the described hPTH is not made synthetically. See U.S. Patent No. 5,010,010 at col. 1, lines 20-27, which corresponds to Applicants' 1986 priority document. Exemplary support for the term "intact" is found at column 2, line 3, of U.S. Patent No. 5,010,010.

A detailed listing of all claims that are, or were, in the application, irrespective of whether the claims remain under examination in the application, is presented, with an appropriate defined status identifier.

Upon entry of this Amendment, claims 31 and 33-42 will remain pending in the application.

Because the foregoing amendments do not introduce new matter, entry thereof by the Examiner is respectfully requested.

II. Response to Issues Raised by Examiner in Outstanding Office Action

A. Claim Rejections - 35 U.S.C. § 102

1. Rejection of Claims 36-42 Under 35 U.S.C. § 102(b) as Anticipated by Brewer et al.

Claims 36-42 are rejected by the Examiner under 35 U.S.C. § 102(b) as being allegedly anticipated by Brewer et al., U.S. Patent No. 3,886,132 ("Brewer"). Applicants respectfully traverse this ground for rejection.

The PTH of Brewer was extracted from cadaveric parathyroid glands. Therefore, the extracted material would necessarily contain human derived proteins and/or human infectious agents. Solely to expedite prosecution, Applicants have amended claim 36 to recite that the hPTH (1-84) is free of human derived proteins and human infectious agents, and does not contain chemically modified amino acids. This is in contrast to the material of Brewer, which necessarily contains human derived proteins and/or human infectious agents. Thus, the disclosure of Brewer falls outside of the scope of claim 36, as amended. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejection.

2. Rejection of Claims 31 and 33-34 Under 35 U.S.C. § 102(b) as Being Anticipated by Breyel

Claims 31 and 33-35 are rejected by the Examiner under 35 U.S.C. § 102(b) as being allegedly anticipated by Breyel et al., "*Synthesis of mature human parathyroid hormone in Escherichia coli*, THIRD EUROPEAN CONGRESS ON BIOTECHNOLOGY 3:363-369 (1984) ("Breyel"). The Examiner asserts that Breyel teaches expression of mature hPTH in *E. coli* and that the extracts would inherently be free of human derived proteins and human infectious agents. Applicants respectfully traverse this ground for rejection.

Solely to expedite prosecution, Applicants have amended claim 31 to recite "[a] preparation of *intact* hPTH (1-84)." The extracts of Breyel do not anticipate claims 31 and 33-35 for the reasons discussed below. Therefore, Applicants respectfully request reconsideration and withdrawal of the rejection.

i. Breyel Does Not Teach Intact hPTH (1-84)

The extracts of Breyel are not intact hPTH(1-84). Specifically, Breyel disclosed expression of hPTH in *E. coli* (see page 363, "Summary," of Breyel). *E. coli* has endogenous exopeptidase and endopeptidase activity which cleaves internal protease sensitive domains in PTH. See Mathavan et al., "High Level Production of Human Parathyroid Hormone in Bombyx mori Larvae and BmN Cells Using Recombinant Baculovirus," *Gene, 167*:33-39, at 34 (1995) (Exhibit 1). As a result, the hPTH of Breyel contains fragments of hPTH. See e.g., page 2, line 34, through page 3, line 8, of the specification, where it is noted that Breyel demonstrated *E. coli* degradation of human PTH. Therefore, claims 31 and 33-35 are not anticipated by Breyel.

ii. The Board of Patent Appeals and Interferences' Decision dated July 2, 2003 Agreed that Breyel does not teach or suggest intact hPTH (1-84)

On pages 8 and 9 of the Board of Patent Appeals and Interferences Decision dated July 2, 2003 ("the BPAI Decision"), the Board addressed this issue. The Board agreed that Breyel does not teach or suggest intact hPTH (1-84). In particular, on page 9, line 3 the BPAI Decision, it states:

> *"[a]s appellants argue, there is no suggestion in either of Breyel or Sung that any intact protein could be obtained. Based on the evidence of record, it is our opinion that, at best, it would have been obvious to try to isolate 'intact protein' from the degraded preparations of Breyel and Sung. 'Obvious-to-try,' however, is not the standard of obviousness under 35 U.S.C. § 103. See In re O'Farrell, 858 F.2d 894, 903, 7 USPQ2d 1673, 1680 (Fed. Cir. 1988). "*

As the Board previously held that Breyel does not anticipate the claimed invention, it is improper for the Examiner to once again reject the claims over this reference under § 102(b).

3. Rejection of Claims 31 and 33-34 Under 35 U.S.C. § 102(b) as Being Anticipated over Mayer[1]

Claims 31 and 33-35 are rejected by the Examiner under 35 U.S.C. § 102(b) as being allegedly anticipated by Mayer, EP 0 139 076 ("Mayer"). The Examiner asserts that Mayer teaches recombinant production of hPTH in *E. coli* and that this preparation would inherently be free of human derived proteins and human infectious agents. Applicants respectfully traverse this ground for rejection.

Solely to expedite prosecution, Applicants have amended claim 31 to recite "[a] preparation of *intact* hPTH (1-84)." The extracts of Mayer do not anticipate claims 31 and 33-35 for the reasons discussed below. Applicants respectfully request reconsideration and withdrawal of the rejection.

The teachings of Mayer are cumulative to the teachings of Breyel. In Mayer, the PTH gene is expressed in *E. coli* using only an initiating methionine. The expression product therefore accumulates within the cell, and is recovered by cell disruption (see page 5, bottom). The recovered products are analyzed, and shown to be reactive with antibodies that bind the N-terminal and two mid-region sites (residues 28-48 and 48-68). Activity was also seen in the adenylate cyclase assay. However, the extracts of Mayer are not intact hPTH(1-84). As discussed above, *E. coli* has endogenous exopeptidase and endopeptidase activity which cleaves internal protease sensitive domains in PTH. See Mathavan et al., "High Level Production of Human Parathyroid Hormone in Bombyx mori Larvae and BmN Cells Using Recombinant Baculovirus," *Gene, 167*:33-39, at 34 (1995) (Exhibit 1). As a result, the hPTH of Mayer contained fragments of hPTH, which can be reactive with antibodies that bind the N-terminal and two mid-region sites and can yield activity in an adenylate cyclase assay. Mayer teaches only that PTH "activity" can be produced, and not that PTH(1-84) can be produced. Therefore, claims 31 and 33-35 are not anticipated by Mayer.

[1] The Examiner refers to an English translation of Mayer throughout the office action. However, the Examiner did not provide a copy of the English translation with the mailing of the Office Action. Therefore, Applicants relied on the UK version of the patent (Exhibit 1).

The comments in the BPAI Decision regarding Breyel not teaching or suggesting intact hPTH(1-84) apply with equal force to Mayer because, as discussed above, the teachings of Mayer are cumulative to the teachings of Breyel.

4. Rejection of Claims 31 and 33-35 Under 35 U.S.C. § 102 (a), (b) and/or (f) as Being Unpatentable Over Applicants' Admission of the Prior Art

Claims 31 and 33-35 are rejected by the Examiner under 35 U.S.C. § 102 (a), (b) and/or (f) as allegedly being unpatentable over Applicants' admission of the prior art. The Examiner asserts that in order for the hPTH standard discussed in the specification on page 7 to have been useful for a comparison, the hPTH standard must have met the limitations of the pending claims. Applicants respectfully disagree and request reconsideration and withdrawal of the rejection.

The Examiner was not persuaded by Applicants' arguments that the synthetic PTH preparations necessarily contain chemically modified amino acids. According to the Examiner, "while protecting groups are used in the process of synthesis, they must be removed to attach subsequent residues." Office Action at page 6. Applicants respectfully disagree and note that it is not necessary for all protecting groups to be removed to attach subsequent residues. For example, the groups protecting the alpha carbon side chain, which are necessary, particularly for the carboxylates such as Asp and Glu, must remain on the growing peptide chain, and are removed only after the peptide is synthesized. Thus, after coupling is completed, the side chain protecting groups and C-terminally attached resin must be removed. It is not realistic to assume that any reaction to remove the protecting groups would progress to 100% to completion. Furthermore, it is highly unlikely that a supplier of reagent grade material would invest the time and resources to pursue the goal of achieving even theoretically complete deprotection. As a result, protecting groups are present on at least some amino acids in of the PTH molecules in the commercially available synthetic PTH preparations. Therefore, the claimed preparation of intact hPTH(1-84) is not anticipated by the hPTH standard discussed in the specification on page 7.

B. Claim Rejections - 35 U.S.C. §§ 102 or 103

Claims 36-42 are rejected by the Examiner under 35 U.S.C. § 102 (a), (b), and/or (f) as being anticipated, or in the alternative, under 35 U.S.C. § 103 as obvious over, by Applicants' own admission of the prior art for the reasons of record with respect to claims 31 and 33-35. Applicants respectfully request reconsideration and withdrawal of the rejection.

Applicants have amended claim 36 to recite that the hPTH (1-84) "is free of human derived proteins and human infectious agents, and does not contain chemically modified amino acids." Therefore, for the reasons discussed above, claims 36-42 are not anticipated or rendered obvious by the hPTH standard discussed in the specification on page 7.

1. Claim Rejections - 35 U.S.C. § 103

Claim 35 is rejected by the Examiner under 35 U.S.C. § 103 as being allegedly obvious over Breyel or Mayer in view of Kaisha et al., GB 2 092 596 ("Kaisha") and Brewer. Applicants respectfully request reconsideration and withdrawal of the rejection.

i. Claim 35 is not Obvious Over the Combined Teachings of Breyel and Kaisha

The Examiner asserts that a person of ordinary skill in the art would use Brewer's or Kaisha's purification method to isolate intact PTH(1-84) from the expression products of Breyel or Mayer. Applicants respectfully disagree. With respect to Breyel and Kaisha, Applicants direct the Examiner's attention to pages 8 and 9 of the BPAI Decision where the Examiner's rejection of the claims under 35 U.S.C. § 103 over the combined teachings of Breyel and Kaisha was reversed. In particular, the BPAI agreed that the Examiner erroneously applied an "obvious-to-try" standard when she asserted that it would be within the skill of the art as taught by Kaisha to purify intact hPTH (1-84) from a degraded protein preparation with a reasonable expectation of success. Therefore, the rejection of claim 35 as obvious over the combined teachings of Breyel and Kaisha should be withdrawn.

Additionally, Applicants disagree with the Examiner's assertion that Breyel teaches a preparation of intact PTH(1-84), as this assertion contradicts the findings of the BPAI. Breyel describes only an attempt at, but not success with, the production of the mature protein. The Breyel material is not intact PTH(1-84).

ii. Claim 35 is Not Obvious Over the Combined Teachings of Breyel and Brewer

The Examiner asserts that "[t]he person of ordinary skill in the art would be easily able to adapt Brewer's method to the bacterial isolates of Breyel or Meyer. The presence of shorter peptides would in no way interfere with the process." Office Action at page 7, lines 8-10. As discussed above, the BPAI stated that the Examiner erroneously applied an "obvious-to-try" standard when she asserted that it would be within the skill of the art as taught by Kaisha to purify intact hPTH (1-84) from a degraded protein preparation with a reasonable expectation of success. The Examiner is also erroneously applying an obvious-to-try standard with respect to removal of human contaminants that would inherently be present in Brewer's preparation, as the PTH of Brewer was extracted from cadaveric parathyroid glands. The BPAI's analysis should apply with equal force to the Examiner's rejection of claim 35 in view of the combined teachings of Breyel and Brewer. Therefore, this rejection should be withdrawn.

iii. Claim 35 is Not Obvious Over the Combined Teachings of Mayer and Kaisha or Mayer and Brewer

As discussed above, the teachings of Mayer are cumulative to the teachings of Breyel. Therefore, for the reasons discussed above, claim 35 is not obvious over the combined teachings of Mayer and Kaisha or Mayer and Brewer. Applicants respectfully request reconsideration and withdrawal of the rejections.

CONCLUSION

The present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

It is acknowledged that the foregoing amendments are submitted after final rejection. However, because the amendments do not introduce new matter or raise new issues, and because the amendments either place the application in condition for allowance or at least in better condition for appeal, entry thereof by the Examiner is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant(s) hereby petition(s) for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

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Gene, 167 (1995) 33–39

GENE 09313

High-level production of human parathyroid hormone in *Bombyx mori* larvae and BmN cells using recombinant baculovirus

(PTH; cDNA; silkworm; cells; osteoblast function tests)

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SUMMARY

A full-length cDNA encoding human parathyroid hormone (hPTH) containing the prepro region was cloned into *Bombyx mori* baculovirus under the control of the polyhedrin promoter and polyadenylation sequences. After transfection and generation of the recombinant baculovirus, hPTH production was examined in silkworm larvae and BmN cell cultures. The larvae synthesized and efficiently secreted the correctly processed and authentic hPTH (9.4 kDa) with no sign of internal degradation. In BmN cells, the major secreted form was the correctly sized protein, but small amounts of degraded hPTH could also be detected in the medium by immunoblotting. Unlike the situation in larvae, prepro-hPTH could also be demonstrated intracellularly in BmN cells. The concentration of hPTH in the larval hemolymph was about 70 mg/l, as compared to approx. 55 μg/l in the medium per 7.5×10^6 cells. Recombinant hPTH (re-hPTH) from the hemolymph was purified by reverse-phase HPLC and subjected to chemical and biological analyses. The authenticity of the purified re-hPTH was confirmed by N-terminal sequencing, amino acid composition and a mass of 9425 Da, close to the theoretical value. The hormone showed high-affinity receptor binding and full biological potency in increasing cellular cAMP.

INTRODUCTION

Human parathyroid hormone (hPTH) is synthesized in the parathyroid glands as a prepro-hormone consisting of 115 aa. During processing, the pre and pro parts of the hormone are sequentially cleaved off resulting in the formation of the mature 84-aa hormone (Cohn and MacGregor, 1981). hPTH (1–84) is secreted in response to a lowering of serum Ca^{2+} ions, and its physiological function is to elevate serum Ca^{2+} and to maintain the calcium and phosphate homoeostasis (Potts et al., 1982; Reeve et al., 1980). Prolonged and intermittent administration of low to medium doses of biologically active hPTH fragment has been shown to vigorously stimulate bone formation in animals and patients with osteoporosis (Reeve et al., 1980; 1991; Bradbeer et al., 1992).

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Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); ACN, acetonitrile; *B.*, *Bombyx*; *Bm*, *B. mori*; BmN, *Bm* ovarian cell line; BmNPV, *Bm* nuclear polyhedrosis virus; bp, base pair(s); Bv, baculovirus; cAMP, cyclic AMP; cDNA, DNA complementary to RNA; cpm, count(s) per minute; DEPC, diethyl pyrocarbonate; HPLC, high-performance liquid chromatography; hPTH, human PTH; *hPTH*, gene (DNA) encoding hPTH; kb, kilobase(s) or 1000 bp; MS, mass spectrometry; LLC-PK$_1$, porcine renal epithelial cell line; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline; pfu, plaque-forming unit(s); PPG, polypropylene glycol(s); PTH, parathyroid hormone; PTHrP, PTH-related protein; re-, recombinant; *S.*, *Saccharomyces*; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; *UTR*, untranslated region(s); wt, wild type.

Considering the potential pharmaceutical importance of the hormone in treatment of bone metabolic disorders, attempts have been made to produce the hormone employing different expression systems such as *Escherichia coli*, *Saccharomyces cerevisiae* and mammalian cells (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Rokkones et al., 1994). The existence of internal protease sensitive domains has made hPTH susceptible to degradation and inactivation (Høgset et al., 1990; Gabrielsen et al., 1990). Thus, an optimalized system for expression of h*PTH* demands a correct processing without aberrant cleavage so that a high production efficiency can be obtained.

In this paper we compare the expression of h*PTH* in BmN cells and *Bombyx mori* (*Bm*) larvae using the *Bm* baculovirus with the polyhedrin promoter and regulatory sequences (Maeda, 1989a,b). The results demonstrate that the cells and the larvae are fully able to recognize the human signal and pro-part of hPTH.

RESULTS AND DISCUSSION

(a) Construction of recombinant virus for h*PTH* expression

Strategies followed for the cloning of full length h*PTH* cDNA (from pPPTH7) including its prepro part into the vector pBm030 is shown in the Fig. 1 and explained in legend. The re-vector pBmPTH84 harbours the full-length h*PTH* cDNA, including the human signal(pre) sequence and its pro part and is controlled by virus regulatory elements. Cotransfection of BmN cells in culture with the plasmid pBmPTH84 DNA and wt viral DNA (BmNPV) resulted in the formation of polyhedrin-negative re-plaques. Upstream and downstream from the cloned h*PTH* cDNA, about 3 kb viral flanking sequences are present, and during cotransfection, these flanking regions will facilitate homologous recombination so that the polyhedrin gene of the wt virus is replaced with the h*PTH* cDNA. After identification and isolation of re-viral plaques they were purified as described in Methods in the legend to Fig. 2. The re-virus were screened and those giving highest expression of h*PTH* were chosen for further experiments.

(b) Production and secretion of hPTH into larval hemolymph

Hemolymph samples from larvae infected with re-virus and collected after 24, 48 and 72 h, showed a time-dependent increase in two peptides (9.4 kDa and 14.3 kDa) which immunoreacted with hPTH antiserum, while hemolymph from wt virus-infected larvae was negative (Fig. 2A, lanes 2, 3 and 4 versus 1). When standard hPTH(1–84) was loaded on the gel, it appeared as a 9.4-kDa form as expected (Fig. 2A, lane 6). However, when the same standard was mixed with the control-hemolymph, the same two different immunoreactive peptides appeared (9.4 kDa and 14.3 kDa) (Fig. 2A, lane 5). Thus, the 14.3-kDa band appeared to be a hPTH-binding protein as also confirmed in subsequent analysis. hPTH production increased during this period and the highest level was obtained after 72 h, whereafter the larvae succumbed to an infection. A semiquantitative estimation of hPTH in hemolymph collected the 3rd day of infection was carried out. Comparing the intensities of immunoreactivity to the different amounts of known hPTH standards as shown in Fig. 2B when different amounts of hemolymph sample was analyzed, it was estimated that 4 µl contained 0.25–0.5 µg hPTH (Fig. 2B, lanes 5, 6 and 7 versus lanes 2, 3 and 4). The non saturable binding properties of the 14.3-kDa band was verified by addition

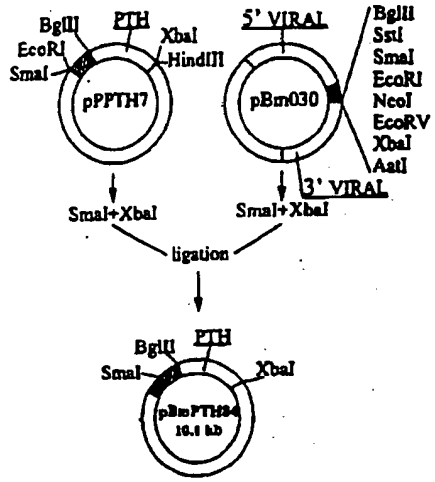

Fig. 1. Construction of baculovirus vector pBmPTH84. The entire h*PTH* cDNA signal and coding sequences were excised from the plasmid pPPTH7 by *SmaI* + *XbaI* digestion and ligated between the *SmaI* and *XbaI* sites of the vector pBm030 (Maeda, 1989b). In the resulting re-vector pBmPTH84 lacking the polyhedrin-encoding gene, the h*PTH* coding sequence is under the control of the polyhedrin promoter, transcriptional termination and polyadenylation sequences, but employing hPTH signal sequence. Methods: Plasmid DNA manipulations were performed essentially as described by Sambrook et al. (1989). A h*PTH* cDNA 413-bp fragment from plasmid pPPTH7 was ligated into vector pBm030, after cleavage with *SmaI* + *XbaI*, and transformed into the *E. coli* DH5α. The clones that contained the h*PTH* cDNA insert were identified and the re-vector named pBmPTH84. The junctions of h*PTH* cDNA and the transfer vector were confirmed by sequencing. Restriction enzymes and other DNA metabolizing enzymes were obtained from New England Biolabs. A anti-rabbit-[^{125}I]IgG was from Amersham. Synthetic hPTH (1–84) from Bachem was used as standard. All the other chemicals used were from Sigma.

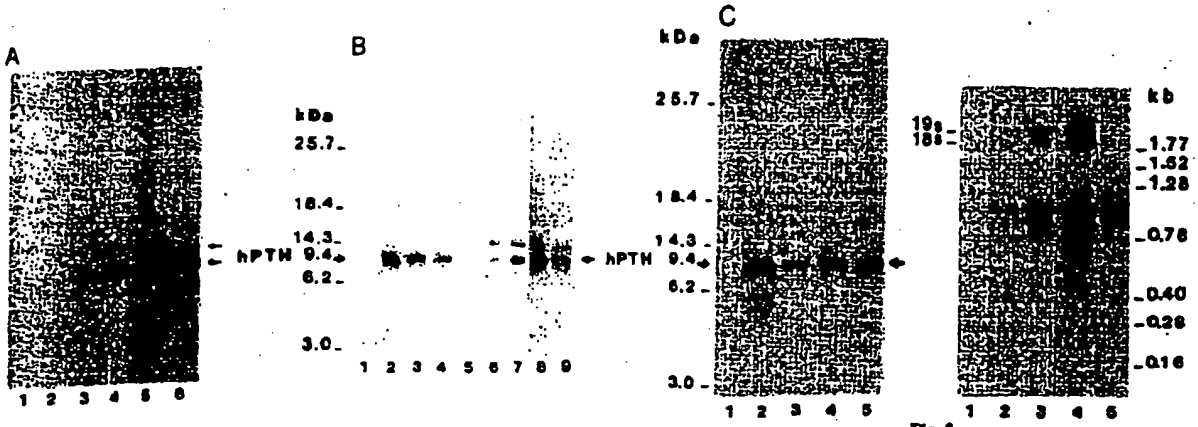

Fig. 2

Fig. 3

Fig. 2. The re-h*PTH* expression. (A) Time-course study of h*PTH* expression in hemolymph from infected larvae collected after 24, 48 and 72 h of infection with re-baculovirus (lanes 2, 3 and 4) compared to control represented by 72 h of wt infection (lane 1) using Western blot technique and mid-region specific anti-hPTH antibody. 3 µl per lane using 0.1% SDS-15% PAGE. Lane 5: hemolymph from wt infected larvae added 0.25 µg hPTH(1–84) standard. Lane 6: 0.25 µg hPTH(1–84) standard. (B) Semiquantitative estimation of hPTH produced in hemolymph 3 days after infection with wt (lane 1 (4 µl)) and re-baculovirus (lanes 5 (1 µl), 6 (2 µl) and 7 (4 µl)) and subjected to 0.1% SDS-15% PAGE, followed by immunoblot analysis. Lanes 2, 3 and 4: hPTH(1–84) marker (Bachem), 0.75 µg, 0.5 µg and 0.25 µg, respectively. Lanes 8 and 9: 4 µl of hemolymph from wt infected larvae added 0.5 µg and 0.25 µg hPTH(1–84) marker, respectively. (C) hPTH in BmN cell cultures 4 days after infection with re-virus (lane 2) and wt virus (lane 1). 5 ml of culture medium partly purified on a Sep-Pak column freeze-dried and analyzed by SDS–PAGE as in panel B. Lanes 3, 4 and 5: 0.05 µg, 0.1 µg and 0.075 µg, respectively, of hPTH(1–84) standard. Methods: *Bm larva and BmN cell culture*: The silkworm *Bm* larvae (TW × NB4D2) were fed ad libitum on fresh mulberry leaves and reared in the laboratory following the method of Krishnaswamy et al. (1973). BmN cells were grown in TC-100 medium containing 10% fetal calf serum and 50 µg gentamycin per ml at 27°C (Maeda, 1989a,b). *Transfection and isolation of re-virus*: Re-vector pBmPTH84 was amplified and purified. Subconfluent monolayers of BmN cells were co-transfected with purified infectious *Bm* wt baculovirus (BmNPV) DNA and the re-vector pBmPTH84. Homologous recombination between the plasmid DNA and wt viral DNA occurred in the Ca-phosphate mediated cotransfected cells as tested after 5 days by plaque assay, and the polyhedrin-negative plaques were screened for hPTH production in the BmN cells and *Bm* larvae. *Collection of larval hemolymph, fatbody, BmN cell culture medium and cell lysate*: Early fifth instar (24 h old) *Bm* larvae were needle inoculated with 50 µl of recombinant viral solution (3×10^5 pfu) into the body cavity using wt virus and saline injections as controls. Hemolymph was collected and treated as described (Maeda, 1989a). BmN cells (7.5×10^6 cells) were seeded in a tissue culture flask and after overnight incubation, the cells were infected by re-virus or wt virus (10 pfu/cell) separately. After 4 days of infection the medium was collected, the samples centrifuged at 1400 rpm for 5 min and the medium and cell pellet stored separately. All the samples were stored at −70°C till further analysis. *Protein determination*: Protein in hemolymph was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. *PAGE and immunoblotting*: 0.1% SDS-15% PAGE was performed under reducing conditions (Laemmli, 1970) and samples were solubilized as previously described (Towbin et al., 1979; Gabrielsen et al., 1990). *Semiquantitative protein determination of hPTH*: Semiquantitative measurements of hPTH using light scanning were performed on X-ray films developed after Western immunoblots using Bio Image system, Millipore.

Fig. 3. mRNA was prepared from fatbody cells and analyzed on agarose gels followed by Northern blot and hybridization to a h*PTH* cDNA fragment. Lanes: 1, 1.3 µg mRNA from wt-infected larvae (control) 72 h post-infection; 2, 3 and 4, 1.3 µg mRNA from re-virus-infected larvae 24, 48 and 72 h after infection; 5, 1 µg of PTH-mRNA isolated from human parathyroid adenomas. Ribosomal RNAs corresponding to 18S and 19S, respectively, are indicated. Methods: Total RNA was extracted from larval fatbody (wt virus infected and re-virus infected) as described (Glisin et al., 1974). Poly(A)$^+$ RNA was selected from identical amounts of total RNA from all the extractions using magnetic oligo(dT) Dynabeads (Dynal A.S. Norway). For time-course response, RNA was extracted at every 24 h post infection for a period of 3 days. The poly(A)$^+$ RNA samples were subjected to electrophoresis on a 1.5% agarose gel containing 6% (v/v) formaldehyde in 20 mM Na-phosphate pH 7.0 buffer. The RNA was subsequently transferred to a nylon membrane by passive diffusion and immobilized by UV light (2 min) and baked at 80°C for 1 h. The h*PTH* cDNA *Xba*I-*Eco*RI fragment was used for probe, and filters were subjected for hybridization at 42°C following standard procedures (Sambrook et al., 1989).

of 0.5 µg and 0.25 µg of hPTH, respectively, to the wt hemolymph (Fig. 2B, lanes 8 and 9) which by itself contained non-detectable immunoreactivity (Fig. 2B, lane 1).

(c) Production and secretion of hPTH by BmN cells into culture medium

BmN cells infected with re-virus also produced and secreted hPTH into the medium. Two secreted proteins were detected on immunoblots of SDS-PAGE using mid-region specific anti-h*PTH* antiserum (Fig. 2C, lane 2). One comigrated with hPTH(1–84) standard (9.4 kDa) and the other and somewhat diffuse 5.5-kDa immunoreactive band probably represented proteolytic degradation products. The amount of hPTH was estimated to be about 0.3–0.5 µg from the flask with an initial concentration of 7.5×10^6 cells as determined by light scanning of the X-ray films developed after various times and compared to hPTH standards (lanes 3, 4 and 5). No immuno-

reactivity was found in medium from wt virus-infected cells (Fig. 2C, lane 1).

(d) The presence of h*PTH* mRNA

The presence of h*PTH* mRNA in fatbody cells was also studied 24, 48 and 72 h after infection and analyzed on agarose gels followed by Northern-blot and hybridization to a *PTH*cDNA *Xba*I-*Eco*RI fragment (Fig. 1) as probe. A time-dependent increase (about 100-fold) in transcripts corresponding in size to *PTH*mRNA prepared from human parathyroid adenomas was observed (Fig. 3, lanes 2, 3 and 4 versus lane 5). In addition, two higher M_r transcripts appeared of sizes equal to 18S and 19S rRNAs.

The presence of three mRNA species in the fat body may indicate heterogeneity within the non-translated regions since only one peptide form was demonstrated. They cannot be due to non-specific hybridization to remaining ribosomal RNA, since wt RNA gave no signal (Fig. 3, lane 1); in addition, a time-dependent increase was also observed.

(e) Intracellular hPTH in larvae and in cultured cells

Intracellular proteins from larval fatbody and BmN cells infected with re-virus were examined using immunoblots and compared to wt virus-infected controls. No hPTH immunoreactivity was detected in the larval fatbody cells while BmN cells showed two dominant hPTH immunoreactive bands. The major one of 13.5 kDa was similar to unprocessed prepro-hPTH while the 16-kDa protein could represent a modified variant or protein bound form of prepro-hPTH (data not shown).

(f) Quantitative measurements of hPTH by two-site chemiluminometric (sandwich) immunoassay

hPTH(1–84) in the hemolymph and culture medium was assayed using chemiluminometric immunoassay according to the manufacturer (Magic Lite, Ciba Corning, Germany). In hemolymph collected three days after coelomic infection, the hPTH concentrations were 0.05–0.1 g/l, while the total protein concentration was 63 g/l. In BmN cell culture medium at day 4 postinfection, the maximal hPTH concentrations were 40–70 µg/l per 10^6 cells.

(g) Reverse-phase HPLC-purification of hPTH and assessment of the chemical purity and authenticity

The re-hPTH was extracted from hemolymph and further purified as described in Methods to Fig. 4. The HPLC purification profiles are shown in Fig. 4 (A, B and C). hPTH from the last HPLC-step (Fig. 4C) was analyzed further on SDS-PAGE (Fig. 4D and E). The results from the gel analyses, including silver-staining (Fig. 4D) and immunoblot analysis (Fig. 4E) showed only one band with a mobility equal to standard hPTH and a purity equal to or better than the hPTH(1–84) Bachem standard (Fig. 4D and 4E, lanes 1 and 3 compared to lanes 2 and 4).

The purified PTH was also subjected to aa composition analysis and N-terminal sequencing which were consistent with the theoretical prediction (data not shown). Mass spectrometry was performed with a spectrum as shown in Fig. 5A and an M_r of 9425 was obtained from the single-charged molecular ions corresponding well

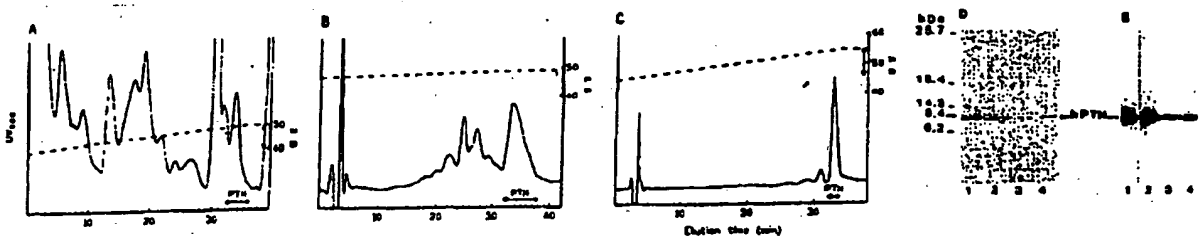

Fig. 4. Separation of re-hPTH(1–84) by reverse-phase HPLC from hemolymph of infected silkworms (A, B and C) and characterization by SDS-PAGE (D and E). (A) Preparative scale chromatography was performed on a Pharmacia SuperPac Pep-S C_{18}/C_2 column (22.5 × 250 mm) as previously described by Olstad et al. (1992) with small modifications. Eluant A, 0.115% TFA in MilliQ water; eluant B, 0.085% TFA in 70% aqueous acetonitrile (ACN) (10 ml/min). (B) Pooled fractions from A) containing hPTH were analyzed on an analytic Pharmacia SuperPac Pep-S C_{18}/C_2 column (4.0 × 250 mm) (linear gradient of 47–49% eluant B run for 35 min). Eluant A and eluant B were the same as in A. The flow rate was 1.0 ml/min. (C) Pooled fractions from B) containing hPTH were further purified on Pharmacia SuperPac Pep-S C_{18}/C_2 column (4.0 × 250 mm). A linear gradient of 45–55% eluant B was run for 35 min. Eluant A, 0.55% TFA in MilliQ water; eluant B, 0.45% TFA in 70% aqueous ACN. The flow rate was 1.0 ml/min. (D and E) 0.1% SDS-15% PAGE analysis of HPLC purified hPTH from hemolymph (Fig. 3C, fractions 33 and 34) and compared to hPTH(1–84) standard from Bachem. (D) Silver-staining. (E) Immunoblot analysis using the mid-region specific anti-hPTH antiserum. Lanes 1 and 3: 1.0 µg and 0.2 µg of hPTH from hemolymph. Lanes 2 and 4: 1.0 µg and 0.2 µg hPTH(1–84) standard (Bachem). Methods: *Purification of hPTH from the medium*: hPTH was concentrated and partly purified as described previously (Olstad et al. 1992) and after freeze-drying, the samples were dissolved in sample-buffer for SDS-PAGE analyses (Laemmli, 1970). *Reverse-phase HPLC*: Preparative and analytical scale chromatography was performed as described previously on SuperPac Pep-S C_{18}/C_2 column (22.5 × 250 mm/4.0 × 250 mm) (Reppe et al., 1991; Olstad et al., 1992). For silver staining of the gel, the procedure for the Sigma silver stain kit was followed.

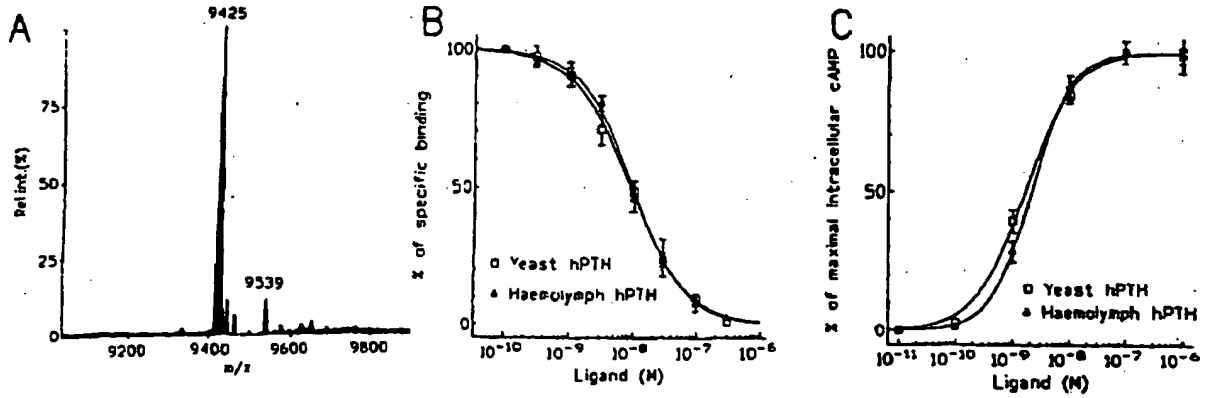

Fig. 5. Characterization of re-hPTH by mass-spectroscopy (A), radioreceptor binding (B) and intracellular cAMP stimulation (C). (A) Mass-spectroscopic analysis of recombinant hPTH(1–84). The dominant single peak represents a molecular hPTH mass of 9425 Da corresponding to the theoretical value of 9424.7 Da. (B) Inhibition of radiolabeled [Tyr^{36}]chicken-PTH-related protein(1–36)amide by different hPTHs. The re-hPTH(1–84) produced in infected silkworms and recombinant hPTH(1–84) (yeast hPTH) produced in *Saccharomyces cerevisiae* were tested in a radioreceptor assay using LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean ± SEM of three independent experiments each performed in triplicate. (C) Stimulation of cellular cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK$_1$ cells transfected with the rat PTH/PTHrP receptor and stimulated (15 min, 37°C) with re-hPTH(1–84) produced in silkworms and re-hPTH(1–84) (yeast hPTH) produced in *Saccharomyces cerevisiae* is shown. The data represent the mean ± SEM of three independent experiments each performed in duplicate. Methods: *Mass spectrometry analysis* was performed using a API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada) and carried out as described (Covey et al., 1988). *Radioreceptor assay*: LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993), were plated in 24-well plates (50 000 cells/well) and grown for 2 days before incubation with ^{125}I-labelled [Tyr^{36}]chicken-PTHrP(1–36)NH$_2$ (100 000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris-HCl, pH 7.7/100 mM NaCl/5 mM KCl/2 mM $CaCl_2$/5% heat-inactivated horse serum/0.5% heat-inactivated fetal calf serum) as described (Jöppner et al., 1988). The competing ligands were recombinant hPTH(1–84) expressed in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and recombinant hPTH(1–84) purified from hemolymph of infected silkworms. Techniques used for radioiodination of PTHrP analog were previously reported. *Intracellular cAMP measurements*. Measurements of intracellular cAMP in LLC-PK$_1$ cells expressing the rat PTH/PTHrP receptor (Bringhurst et al., 1993) using Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min, then washed and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 M HCl. The stimulating ligands were re-hPTH(1–84) produced in yeast (yeast hPTH) (Gabrielsen et al., 1990; Olstad et al., 1992) and re-hPTH(1–84) produced in *Bm* hemolymph.

with the theoretical M_r of 9424.7 for hPTH as calculated from the aa composition.

(h) Radioreceptor binding studies and intracellular cAMP measurements

Binding properties of two different re-hPTH forms from yeast and silkworm are shown in terms of displacement curves using the ^{125}I-labeled [Tyr^{36}]chicken-PTHrP(1–36)NH$_2$ as radioligand and LLC-PK$_1$ cells permanently transfected with the rat PTH/PTHrP receptor. Both hPTH forms representing the authentic hormones, showed equal receptor binding affinities (Fig. 5B) (calculated $K_d = 8.8 \pm 1.2 \times 10^{-9}$ M) and identical abilities to stimulate intracellular cAMP accumulation in the same cells with half maximal response obtained at 2.0×10^{-9} M (Fig. 5C).

(i) Concluding remarks and comparison of h*PTH* expression between various host systems

The quantitative result of re-hPTH production in BmN ovarian cell culture was much less than that in the hemolymph even corrected for the 30%–50% loss during medium concentration and Sep-Pak column chromatography (see Methods in the legend to Fig. 4). The circulatory system of silkworm larvae opens into the coelomic cavity which is totally bathed in the hemolymph and retains the secretory proteins (Shigematsu, 1958).

hPTH could not be detected in the intracellular fractions of larval fatbody while the polypeptide and the assumed unprocessed forms were present in BmN cells. Ovarian cells are normally not designed for secretion of proteins, but rather for absorption, and this may be a reason for the low level of hPTH produced. The hormone and its mRNA were expressed in a parallel and a time-dependent fashion. The hPTH produced in the silkworm larvae was authentic as judged by N-terminal sequence, total aa composition and mass spectrometry. Also its receptor binding affinity and ability to activate the main second messenger system were identical to the yeast re-hPTH which previously was shown to have full biological activity in several target cell systems (Reppe et al., 1991).

The amount of hormone produced in the larval hemolymph represented 70 mg/l. This level was many times

higher than that reported for yeast (Gabrielsen et al., 1990; Reppe et al., 1991) and for *E. coli* (Høgset et al., 1990) secreted hPTH. The re-hPTH produced as an intracellular fusion protein with *S. aureus* protein A, showed a production after purification of 50–80 mg/l culture (Forsberg et al., 1991).

The production yield of hPTH in the silkworm larvae also compared well to ZZ-cecropin A fusion protein production in *Trichoplusia ni* larvae using *Autographa californica* baculovirus (Andersons et al., 1991). Production of human α-interferon in silkworm using *Bm* baculovirus, amounted to 30 mg/l hemolymph after purifying 10 ml of hemolymph by affinity column chromatography (Maeda et al., 1985). However, they did not report the concentration of α-interferon in the hemolymph prior to purification.

Expression of h*PTH* in microbiological systems has met with two problems related to incorrect N-terminal cleavage and aberrant intracellular processing (Rabbani et al., 1988; Høgset et al., 1990; Gabrielsen et al., 1990; Reppe et al., 1991; Forsberg et al., 1991; Rokkones et al., 1994). The major cleavage sites were after Lys^{26} in *S. cerevisiae* and after Val^{21} in *E. coli* (Rokkones et al., 1994). In mammalian cells, in contrast (mouse mammary tumor cell, i.e., C1271 cells, and Chinese hamster lung cells, i.e., DON cells) the entire h*PTH* cDNA including the prepro part gave rise to only the intact form (Rokkones et al., 1994). We demonstrate that the silkworm larvae in fact resembles the mammalian system cleaving the hPTH signal sequence correctly and that the human signal is also able to promote an efficient secretion of the intact hormone. However, the hPTH produced binds to a natural protein in the hemolymph and gives rise to a 14.3-kDa protein in addition to the expected 9.4-kDa form. The N-terminal sequence of the purified '14.3-kDa protein' was identical to hPTH(1–84) (data not shown). Standard hPTH also showed the same two bands when added to the hemolymph (Fig. 2A and B). Moreover, in buffer containing urea, the mobility of the 14.3-kDa form was normalized (data not shown) and also the 'acid treatment' occurring during HPLC purification released the peptide. Thus, the '14.3-kDa protein band' represents a hPTH-binding protein of unknown nature.

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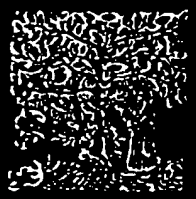

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Date of Publication of the Translation 04 NOV 1992

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IN THE MATTER OF

European Patent (UK)

No. 0 139 076

I, JUDITH MARGARET ATKINSON, B.A., A.I.T.I., of 56 Hollins Grove Street, Darwen, Lancashire, do hereby declare that I am the translator of European Patent Specification No. 0 139 076 and certify that the following is a true translation to the best of my knowledge and belief.

J.M. Atkinson.

Dated this 28th day of September 1992

Human parathyroid hormone regulates, inter alia, the incorporation and release of calcium in bones. The effects and functions of human parathyroid hormone and its agonists and antagonists are described by Dambacher, Praktische Osteologie (Practical Osteology), Thieme Verlag Stuttgart/New York 1982; Reeve et al., British Medical J. 1340 (1980) 1-11; and Potts et al., Advances in Protein Chemistry, 35 (1982) 323-395.

In Int. Congr. Ser.-Excerpta Med., (1980) 5-18, the expression of Pre-Pro-bPTH and Pro-bPTH is described. In Proc. Natl. Acad. Sci. USA, 78 (1981) 7365-7369, the DNA sequence of hPre-Pro-PTH is described. This description was not, however, sufficient to express biologically active hPTH; cf. Mol. Biol. Radiochem. Essays Calciotropic Horm. Sattelite Symp., (1984) p. 80287.

The problem underlying the invention is to make available biological material with which human parathyroid hormone (hPTH) can be manufactured industrially.

In accordance with the present invention, this problem is solved by a hybrid vector which can be cloned in prokaryotic cells and which produces human parathyroid hormone and is characterised by the following features:
(a) a promoter,
(b) a DNA region, adjacent to the promoter, of from 0 to 1000 or from 0 to 200 base pairs,
(c) a ribosome binding site, adjacent to the DNA region according to (b) or, where (b) is lacking, adjacent to the promoter according to (a),
(d) a DNA region, adjacent to the ribosome binding site, of from 4 to 15 base pairs,
(e) a start codon, adjacent to the DNA region according to (d), and
(f) the following DNA sequence encoding human parathyroid

hormone:

```
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His
TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT
AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG GAC CCT TTT GTA


Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu
CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG
GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA TTC TTC GAC


Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro Leu Ala
CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT
GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA GAT CGA


Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu Asp
CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA GAC
GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT CTG


Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT


Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG T
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC A
```

As prokaryotic cells there come into consideration all cells in which it is possible to clone hybrid vectors having the features indicated on an industrial scale with the formation of human parathyroid hormone. Details regarding cloning and expression of genes under the control of E. coli promoters (for example in Streptomyces) can be found in Chater, Nature, 299 (1982) 10 ff. Escherichia coli is especially suitable. Examples of suitable promoters for E. coli can be found, for example, in Sengbusch, P. von, Molekular- und Zellbiologie (Molecular and Cell Biology), Springer-Verlag, Heidelberg etc., 1979. In the case of E. coli the ribosome binding site (RB) can have, for example, the following DNA sequence:

AGGA or GGAG
TCCT CCTC

An example of a start codon that can be used in the case of E. coli has the DNA sequence:
ATG
TAC

According to a special embodiment of the invention, the specific DNA sequence according to (f) may be replaced by a DNA sequence the single strands of which can be hybridised with the single strands of the specific DNA sequence, the replacement DNA sequence likewise expressing human parathyroid hormone.

According to a further special embodiment of the invention, in the specific DNA sequence according to (f) one or more base triplets may be replaced by base triplets that encode the same amino acid.

In the following, the manufacture of the hybrid vector according to the invention is described in more detail with reference to an Example and three schemes.

Example

The parathyroids were removed from freshly slaughtered pigs. mRNA was isolated from this glandular tissue. The isolated mRNA was cloned in E. coli as a double-strand complementary DNA (ds-cDNA) with the aid of a plasmid. The hybrid plasmid-DNA was isolated from the resulting hybrid clones and fixed in its single-strand form to a carrier. mRNA isolated from the parathyroids was hybridised with fixed single-strand DNA and removed and translated in an <u>in vitro</u> translation system into pig Pre-Pro-parathyroid hormone or pig parathyroid hormone.

The pig Pre-Pro-parathyroid hormone or pig parathyroid hormone was demonstrated by antibody precipitation. With the aid of "hybrid arrested translation" and DNA sequence analysis it was possible to determine the clones that contained the pig parathyroid hormone sequences. From the hybrid clones determined, hybrid plasmid-DNA that included the pig cDNA parathyroid hormone gene (Scheme 1) was radioactively labelled (nick-translated) and used for screening human gene banks. Human parathyroid hormone gene determined in this manner was enriched by subcloning in a plasmid. A human parathyroid hormone gene enriched in this manner was sequenced. The sequence of the section relevant for the expression of human parathyroid hormone can be seen in Scheme 2. The sequence determined agreed with the known cDNA sequence and with the known amino acid sequence of human parathyroid hormone.

Scheme 3 is explained in the following. (1) In the further process there was used the DNA sequence located between two EcoRI incision sites, which DNA sequence includes the DNA sequence of Scheme 2. With the aid of the restriction endonuclease Sau3A the Pre-Pro part of the PTH gene was then separated from the gene of the mature 1-84-PTH (2). By filling up dATP and dGTP using the "large fragment" of E. coli-DNA-polymerase-I (3) and subsequently digesting with S1-nuclease, the remaining single-strand residue (GA) of the sticky ends (GATC), which originated from the Sau3A cleavage, was eliminated; as a result the codon "TCT" for amino acid 1 (serine) of human PTH was reconstituted (4). A DNA adaptor was linked to this PTH-DNA-fragment treated in the manner indicated. As a result, a methionine codon "ATG" was connected directly before the serine. This codon is one of the important signals for the start of the synthesis of PTH in the microorganism (5). The PTH gene fragment

constructed in the indicated manner was subcloned into the ClaI-cleavage site of pBR322. A clone was selected in which the PTH gene was oriented in front of the HindIII cleavage site of the pBR322 (6 to 7) in the anti-clockwise direction. This PTH clone was cleaved with HindIII (7). The "sticky ends" of this cleavage site allowed filling-up reactions with four different nucleotides. In combination with digestion by S1-nuclease, there were obtained fragments having filled-up ends (blunt ends) with a distance from the ATG codon of 4 to 10 base pairs (8). In front of this variation of fragments there were linked two synthetic DNA adaptors, these being /TCCCTAGGGA/+/TCCCTAGGGA/ (9). These linkers contained the sequence of the ribosome binding site, a further important signal for expression in the micro-organism. As a result there was formed also a BamHI cleavage site (10). This was suitable for cloning after various promoters (such as trp, tac, T5) of described vectors (11).

The transformed E. coli cells were grown in LB complete medium in the presence of ampicillin (50 µg/ml) until the central logarithmic phase was reached, and were centrifuged off. The pellet was suspended in a suspension buffer containing guanidinium hydrochloride (3M) (approximately 10^{10} cells/ml); thereafter disintegration was effected with ultrasound (sonifier) until an optical density (OD_{650}) was reached which corresponded to approximately 1/3 of the optical density at the beginning. This cell debris was centrifuged off. The supernatant contained PTH. The protein was precipitated with 5 % TCA and dissolved in 0.02N hydrochloric acid. TCA residues were washed out with ether. The PTH obtained from the crude extract and after extraction was immunologically active against antibodies (specific to PTH fragments AS1-34, AS28-48 and AS48-68) (RIA) and was

biologically active in the adenylcyclase test.

The human parathyroid hormone gene was linked to a vector (pBR322/SV40 derivative) and transformed with the aid of calcium phosphate precipitation into monkey kidney cells. From 10^9 cells, PTH was obtained by extraction; in the RIA it was positive against antibodies (specific to the PTH fragments AS28-48 and AS44-68).

In addition, the human parathyroid hormone gene (λ-human hybrid-DNA) was transformed with the thymidine kinase gene of Herpes simplex by co-transformation into T3 cells; from the Tk^+ clones the integration of human PTH gene was identified by DNA hybridisation.

Scheme 1

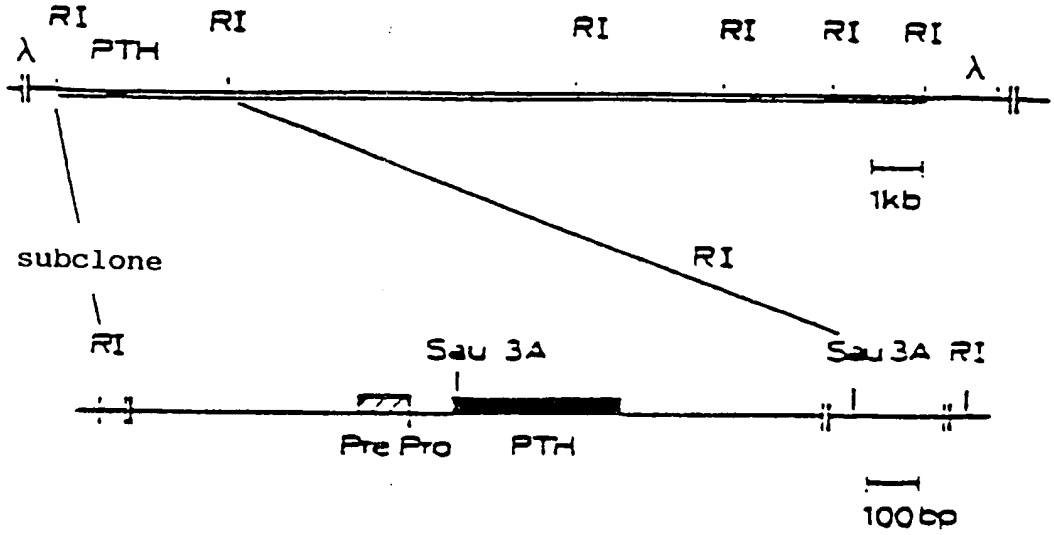
```
Asp Thr Val Lys Val Met Val Val Met Leu Ala Ile Cys Phe
GAC ACA GTT AAA GTA ATG GTT GTC ATG CTT GCA ATT TGT TTT
CTG TGT CAA TTT CAT TAC CAA CAG TAC GAA CGT TAA ACA AAA

Leu Ala Arg Ser Asp Gly Lys Pro Val Lys Lys Arg Ser Val
CTT GCA AGA TCA GAT GGG AAG CCT GTT AAG AAG AGA TCT GTG
GAA CGT TCT AGT CTA CCC TTC GGA CAA TTC TTC TCT AGA CAC

Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His Leu Ser
AGT GAA ATA CAG CTT ATG CAT AAC CTG GGC AAA CAC CTG AGC
TCA CTT TAT GTC GAA TAC GTA TTG GAC CCG TTT GTG GAC TCG

Ser Leu Glu Arg Val Glu Trp Leu Arg Lys Lys Leu Gln Asp
TCT CTG GAG AGA GTG GAA TGG CTG CGA AAG AAG CTG CAG GAT
AGA GAC CTC TCT CAC CTT ACC GAC GCT TTC TTC GAC GTC CTA

Val His Asn Phe Val Val Leu Gly Ala Ser Ile Val His Arg
GTG CAC AAC TTT GTT GTT CTC GGA GCT TCT ATA GTT CAC AGA
CAC GTG TTG AAA CAA CAA GAG CCT CGA AGA TAT CAA GTG TCT

Asp Gly Gly Ser Gln Arg Pro Pro Lys Lys Glu Asp Asn Val
GAT GGT GGT TCC CAG AGA CCC CCA AAA AAG GAA GAC AAT GTC
CTA CCA CCA AGG GTC TCT GGG GGT TTT TTC CTT CTG TTA CAG

Leu Val Glu Ser His Gln Lys Ser Leu Gly Glu Ala Asp Lys
CTA GTT GAG AGC CAT CAA AAA AGT CTC GGA GAA GCA GAT AAA
GAT CAA CTC TCG GTA GTT TTT TCA GAG CCT CTT CGT CTA TTT

Ala Ala Val Gly
GCT GCT GTG GGG
CGA CGA CAC CCC
```

```
TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

CATTGTAT
GTAACATA                    Intron II ca. 400 bp

                  Met Ile Pro Ala Lys Asp Met Ala Lys Val Met
        GTG AAG ATG ATA CCT GCA AAA GAC ATG GCT AAA GTT ATG
        CAC TTC TAC TAT GGA CGT TTT CTG TAC CGA TTT CAA TAC

Ile Val Met Leu Ala Ile Cys Phe Leu Thr Lys Ser Asp Gly Lys
ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA AAA TCG GAT GGG AAA
TAA CAG TAC AAC CGT TAA ACA AAA GAA TGT TTT AGC CTA CCC TTT

Ser Val Lys
TCT GTT AAG
AGA CAA TTC

Intron I      Lys Arg Ser Val Ser Glu Ila Gln Leu Met His Asn
ca. 80 bp     AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
              TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG

Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg
CTG GGA AAA CAT CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT
GAC CCT TTT GTA GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA

Lys Lys Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro
AAG AAG CTG CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT
TTC TTC GAC GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA

Leu Ala Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu
CTA GCT CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA
GAT CGA GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT

Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
CTG TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT

Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG TGA
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC ACT

AAA TGA AAA CAG ATA TTG TCA GAG TTC TGC TCT AGA CAG TGT AGG
TTT ACT TTT GTC TAT AAC AGT CTC AAG ACG AGA TCT GTC ACA TCC

GCA ACA ATA CAT GCT GCT AAT TCA AAG CTC TAT TAA GAT TTC CAA
CGT TGT TAT GTA CGA CGA TTA AGT TTC GAG ATA ATT CTA AAG GTT

GTG CCA ATA TTT CTG ATA TAA CAA ACT ACA TGT AAT CCA TCA CTA
CAC GGT TAT AAA GAC TAT ATT GTT TGA TGT ACA TTA GGT AGT GAT

GCC ATG ATA ACT GCA ATT TTA ATT GAT TAT TCT GAT TCC ACT TTT
CGG TAC TAT TGA CGT TAA TAA TAA CTA ATA AGA CTA AGG TGA AAA

ATT CAT TTG AGT TAT TTT AAT TAT CTT TTC TAT TGT TTA TTC TTT
TAA GTA AAC TCA ATA AAA TTA ATA GAA AAG ATA ACA AAT AAG AAA

TTA AAG TAT GTT ATT GCA TAA TTT ATA AAA GAA TAA AAT TCG ACT
AAT TTC ATA CAA TAA CGT ATT AAA TAT TTT CTT ATT TTA AGC TGA

TTT AAA CCT CTC TTC TAC CTT AAA ATG TAA AAC AAA AAT GTA ATG
AAA TTT GGA GAG AAG ATG GAA TTT TAC ATT TTG TTT TTA CAT TAC

ATC ATA AGT CTA AAT AAA TGA AGT ATT TCT CAC TCA AA
TAG TAT TCA GAT TTA TTT ACT TCA TAA AGA GTG AGT TT
```

Scheme 3

Cloning scheme for human PTH gene expression in E. coli

1) λ -human hybrid



DNA sequence within this region cut out using RI:
(following page)

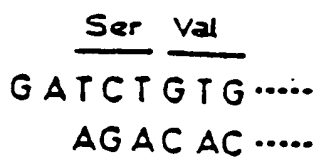
```
TGTCTTTAGTTTACTCAGCATCAGCTACTAACATACCTGAACGAAGATCTTGTTCTAAGA.
ACAGAAATCAAATGAGTCGTAGTCGATGATTGTATGGACTTGCTTCTAGAACAAGATTCT

CATTGTAT
GTAACATA
```

Intron II ca. 400 bp

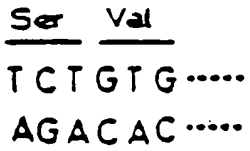
```
                Met Ile Pro Ala Lys Asp Met Ala Lys Val Met
        GTG AAG ATG ATA CCT GCA AAA GAC ATG GCT AAA GTT ATG
        CAC TTC TAC TAT GGA CGT TTT CTG TAC CGA TTT CAA TAC

Ile Val Met Leu Ala Ile Cys Phe Leu Thr Lys Ser Asp Gly Lys
ATT GTC ATG TTG GCA ATT TGT TTT CTT ACA AAA TCG GAT GGG AAA
TAA CAG TAC AAC CGT TAA ACA AAA GAA TGT TTT AGC CTA CCC TTT

Ser Val Lys
TCT GTT AAG
AGA CAA TTC

Intron I        Lys Arg Ser Val Ser Glu Ile Gln Leu Met His Asn
ca. 80 bp       AAG AGA TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC
                TTC TCT AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG

Leu Gly Lys His Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg
CTG GGA AAA CAT CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT
GAC CCT TTT GTA GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA

Lys Lys Leu Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro
AAG AAG CTG CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT
TTC TTC GAC GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA

Leu Ala Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu
CTA GCT CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA
GAT CGA GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT

Asp Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
GAC AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
CTG TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT

Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG TGA
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC ACT

AAA TGA AAA CAG ATA TTG TCA GAG TTC TGC TCT AGA CAG TGT AGG
TTT ACT TTT GTC TAT AAC AGT CTC AAG ACG AGA TCT GTC ACA TCC

GCA ACA ATA CAT GCT GCT AAT TCA AAG CTC TAT TAA GAT TTC CAA
CGT TGT TAT GTA CGA CGA TTA AGT TTC GAG ATA ATT CTA AAG GTT

GTG CCA ATA TTT CTG ATA TAA CAA ACT ACA TGT AAT CCA TCA CTA
CAC GGT TAT AAA GAC TAT ATT GTT TGA TGT ACA TTA GGT AGT GAT

GCC ATG ATA ACT GCA ATT TTA ATT GAT TAT TCT GAT TCC ACT TTT
CGG TAC TAT TGA CGT TAA AAT TAA CTA ATA AGA CTA AGG TGA AAA

ATT CAT TTG AGT TAT TTT AAT TAT CTT TTC TAT TGT TTA TTC TTT
TAA GTA AAC TCA ATA AAA TTA ATA GAA AAG ATA ACA AAT AAG AAA

TTA AAG TAT GTT ATT GCA TAA TTT ATA AAA GAA TAA AAT TCG ACT
AAT TTC ATA CAA TAA CGT ATT AAA TAT TTT CTT ATT TTA AGC TGA

TTT AAA CCT CTC TTC TAC CTT AAA ATG TAA AAC AAA AAT GTA ATG
AAA TTT GGA GAG AAG ATG GAA TTT TAC ATT TTG TTT TTA CAT TAC

ATC ATA AGT CTA AAT AAA TGA AGT ATT TCT CAC TCA AA
TAG TAT TCA GAT TTA TTT ACT TCA TAA AGA GTG AGT TT
```

2) Further cleavage (Sau3A)

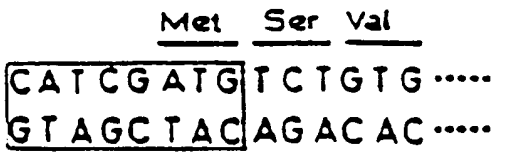
3) Filling up with dG and dA



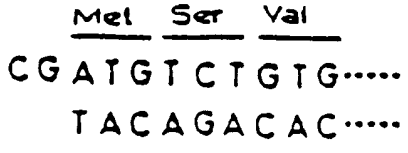
4) Digestion with S1 nuclease



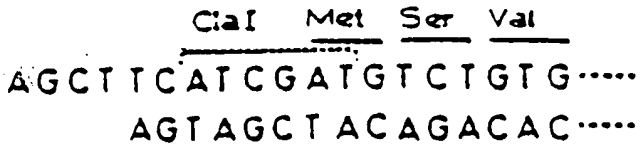
5) Linking with DNA adaptor



6) Further cleavage (ClaI)



7) Subcloning into the ClaI cleavage site of pBR322 and further cleavage (HindIII)



8) various filling-up reactions

Met
TCATCGATG·····
AGTAGCTAC·····

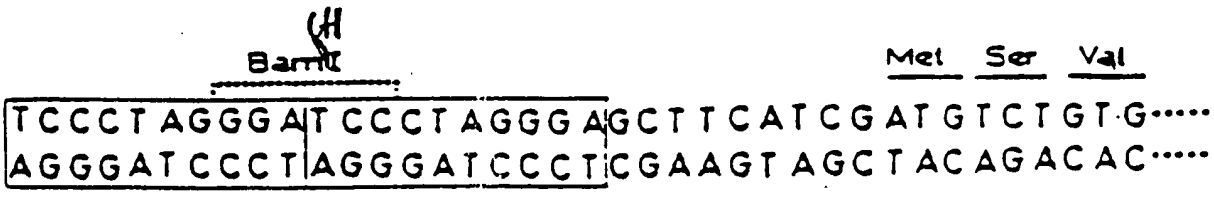
Met
TTCATCGATG·····
AAGTAGCTAC·····

Met
CTTCATCGATG·····
GAAGTAGCTAC·····

Met
GCTTCATCGATG·····
CGAAGTAGCTAC·····

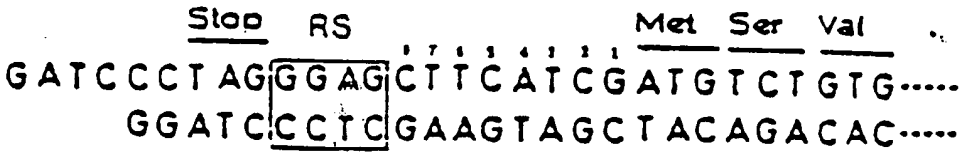
Met
AGCTTCATCGATG·····
TCGAAGTAGCTAC·····

9) Linking with DNA adaptors (2 identical linkers)

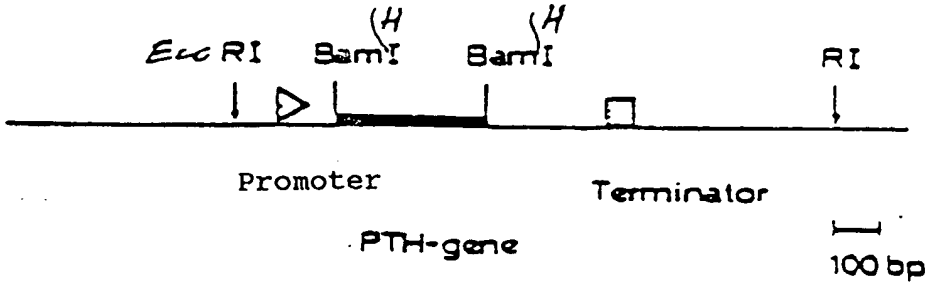


10) Further cleavage (BamHI)

RS = ribosome binding site



11) Insertion into a plasmid behind a strong promoter



Patent claims for BE, CH, DE, FR, GB, IT, LI, SE

1. Hybrid vector which can be cloned in prokaryotic cells and which produces human parathyroid hormone, characterised by the following features:
(a) a promoter,
(b) a DNA region, adjacent to the promoter, of from 0 to 1000 or from 0 to 200 base pairs,
(c) a ribosome binding site, adjacent to the DNA region according to (b),
(d) a DNA region, adjacent to the ribosome binding site, of from 4 to 15 base pairs,
(e) a start codon, adjacent to the DNA region according to (d), and
(f) the following DNA sequence encoding human parathyroid hormone:

```
Ser Val Ser Glu Ile Gln Leu Met His Asn Leu Gly Lys His
TCT GTG AGT GAA ATA CAG CTT ATG CAT AAC CTG GGA AAA CAT
AGA CAC TCA CTT TAT GTC GAA TAC GTA TTG GAC CCT TTT GTA

Leu Asn Ser Met Glu Arg Val Glu Trp Leu Arg Lys Lys Leu
CTG AAC TCG ATG GAG AGA GTA GAA TGG CTG CGT AAG AAG CTG
GAC TTG AGC TAC CTC TCT CAT CTT ACC GAC GCA TTC TTC GAC

Gln Asp Val His Asn Phe Val Ala Leu Gly Ala Pro Leu Ala
CAG GAT GTG CAC AAT TTT GTT GCC CTT GGA GCT CCT CTA GCT
GTC CTA CAC GTG TTA AAA CAA CGG GAA CCT CGA GGA GAT CGA

Pro Arg Asp Ala Gly Ser Gln Arg Pro Arg Lys Lys Glu Asp
CCC AGA GAT GCT GGT TCC CAG AGG CCC CGA AAA AAG GAA GAC
GGG TCT CTA CGA CCA AGG GTC TCC GGG GCT TTT TTC CTT CTG

Asn Val Leu Val Glu Ser His Glu Lys Ser Leu Gly Glu Ala
AAT GTC TTG GTT GAG AGC CAT GAA AAA AGT CTT GGA GAG GCA
TTA CAG AAC CAA CTC TCG GTA CTT TTT TCA GAA CCT CTC CGT

Asp Lys Ala Asp Val Asn Val Leu Thr Lys Ala Lys Ser Gln
GAC AAA GCT GAT GTG AAT GTA TTA ACT AAA GCT AAA TCC CAG T
CTG TTT CGA CTA CAC TTA CAT AAT TGA TTT CGA TTT AGG GTC A
```

2. Hybrid vector according to claim 1, characterised in that it can be cloned in E. coli.

3. Hybrid vector according to claim 1 or 2, characterised in that the specific DNA sequence according to claim 1(f) is replaced by a DNA sequence the single strands of which can be hybridised with the single strands of the specific DNA sequence, the replacement DNA sequence being able to express desired products.

4. Hybrid vector according to claim 1, 2 or 3, characterised in that one or more base triplets are replaced by synonyms.